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(54) Title: METHOD FOR INHIBITION OF CELL ADHESION TO RECEPTORS CONTAINING SELECTINS

(57) Abstract

A method for inhibition of adhesion of cells to receptors containing selectins in mammals is disclosed whereby an anti-adhesion enzyme is administered to the mammal in a therapeutically effective dosage. In a preferred embodiment, the anti-adhesion enzyme will be administered to treat a chronic or acute inflammatory condition (preferably the latter). In this context, the therapeutically effective dosage will be a dosage sufficient to achieve detectable reduction of the inflammation without substantial toxicity. The enzyme administered will specifically cleave carbohydrate residues which are involved in forming bonds between carbohydrate residues in ligands (typically on leukocytes) which are specific for selectin-containing receptors (typically on endothelial cells), inparticular, bonds formed by fucosylated and/or sialilated residues. The enzyme will preferably be a fucosidase or sialidase and is preferably used in recombinant form. Means are also described whereby the activity of the enzyme can be enhanced to perform optimally at pH 7-7.4 (i.e., the pH of plasma). The enzyme may also be modified to extend its *in vivo* half-life and shelf life. It is expected that the same enzyme will cleave many adhesion residues.

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# METHOD FOR INHIBITION OF CELL ADHESION TO RECEPTORS CONTAINING SELECTINS

## BACKGROUND OF THE INVENTION

This is a Continuation-In-Part of U.S. Patent Application Serial No. 07/994,650, filed December 22, 1992, now abandoned.

### 1. *Field of the Invention*

The invention relates to a therapeutic method for the inhibtion of cell adhesion to cell receptors which contain selectins in mammals, particularly in humans. In particular, it relates to a method of interfering with the adhesion of leukocytes to endothelial cell receptors via specific binding of a selectin in the receptor to its corresponding ligand, such as sialilated and fucosylated ligands. More generally, the invention relates to the prevention of adhesion of any cell via interaction between carbohydrate residues in a cell surface ligand to receptors that contain selectins which will specifically bind such carbohydrate residues.

### 2. *History of the Invention*

Binding of inflammatory cells to the endothelial cells which line blood vessels is a necessary prerequisite to generation of an inflammatory reaction to infection or injury in mammals. Inflammation resulting from the adhesion of neutrophils (phagocytic leukocytes found principally in the blood stream) is known to play a role in acute conditions such as myocardial infarction, stroke, septic shock, anaphylactic reactions to drugs and allergens, and the "post-pump syndrome" which can develop following performance of a cardio-pulmonary bypass. Inflammation is also a major cause of

disability in chronic conditions such as rheumatoid arthritis, psoriasis, inflammatory bowel disease and chronic obstructive pulmonary disease. Cell adhesion is also important to metastasis of tumors.

Recently, researchers have proposed that human inflammatory cells adhere to the endothelium of blood vessels via specific endothelial cell receptor-leukocyte ligand interactions involving cell-type specific carbohydrate structures and linkages. A family of adhesion receptor glycoproteins critical to endothelium binding of leukocytes is known as the selectins. One of the selectins in particular (ELAM-1) is thought to be synthesized by endothelial cells in response to the presence of inflammatory agents. ELAM-1 is brought to the cell surface in its synthesis and serves as a receptor for corresponding carbohydrate adhesion ligands (hereafter, "selectin adhesion ligands") on the cell surface of leukocytes.

Analysis of the adhesion ligands for ELAM-1 and other selectins has indicated that the ligand for ELAM-1 contains carbohydrate residues which are part of a family of sialilated fucosylated polylactosamines, a critical determinant in which is sialyl - 2,3Gal $\beta$1,4 [fucose $\alpha$ 1,3] GlcNAc (hereafter, "sialyl Lewis X"). Sialyl Lewis X and Lewis X are $\alpha$ (1→3) fucosylated derivatives of polylactosamines found at the non-reducing termini of glycolipids. Glycolipids and glycoproteins that contain $\alpha$-L-fucose can be hydrolyzed by a lysosomal enzyme known as $\alpha$-L-fucoside fucohydrolase (EC# 3.2.1.51), hereafter $\alpha$-L-fucosidase. Oligosaccharides, gangliosides and glycoproteins which contain sialic acid residues can also be hydrolyzed by a family of enzymes known as the sialidases (EC# 3.2.1.18; also known as neuraminidase).

It has also been suggested that certain tumor cells may metastasize through via cell adhesion events mediated by carbohydrate containing ligand/ selectin containing receptor interactions. It can be expected, therefore, that fucosidases and sialidases will likely also cleave carbohydrate residues involved in metastasis of such tumor cells.

It is the concept of this invention that, if producible in sufficient quantities of consistent quality, administration of enzymes which will specifically cleave carbohydrate residues in selectin adhesion ligands (hereafter collectively, "anti-adhesion enzymes") in therapeutically effective amounts can block cell adhesion. Such a therapeutic modality would be a significant improvement over current models proposed for blocking cell adhesion to reduce inflammation.

For example, in one current model, cell adhesion is blocked by use of monoclonal antibodies specific to sialyl Lewis X or, as one set of researchers have attempted (Goelz, S.E., *et al., Cell* 63:1349-1356 (1990)) by use of a monoclonal antibody specific to an undefined carbohydrate structure in the selectin ligand. Another approach inhibits cell adhesion by the use of soluble receptors (difucosylated sialyl Lewis X glycolipid or sialyl Lewis X-containing mucin) as competitors for binding of the natural ligand. More recently, researchers have shown that lung vascular endothelial injury associated with inflammation induced in adult respiratory distress syndrome (ARDS) can be minimized in rats by administration of an anti-P-selectin monoclonal antibody (Mulligan, *et al., J.Clin.Invest.* 90:1600-1607 (1992)).

While these approaches have been effective to varying degrees in vitro, they appear to have little potential utility for therapeutic purposes because very high concentrations of antibody or soluble receptors must be used to effectively compete with the natural ligand *in vivo*. Further, it is believed that there are a substantial number of selectin adhesion ligands active in inflammatory responses; therefore, a monoclonal antibody or other specific antiligand will not have the broad range of effectiveness of an enzyme specific for carboydrate moieties in selectin adhesion ligands. A need, therefore, exists for safe and effective cell adhesion inhibitors that target carbohydrate residues in selectin adhesion ligands (particularly the sialilated and fucosylated ligands). The present invention addresses that need.

## SUMMARY OF THE INVENTION

The invention is a method for preventing or reducing adhesion of cells mediated by binding of a selectin adhesion ligand to an receptor containing selectin. Preferably, the method involves the administration of a therapeutically effective dosage of an anti-adhesion enzyme (in particular, a sialidase and/or a fucosidase) to a mammal (preferably a human) with acute or chronic inflammation to cleave carbohydrate residues in the ligand, thus preventing adherence of the leukocytes to selectin-containing endothelial cell receptors. It will be appreciated that given the diversity of structure inherent in carbohydrates, other structural components of the selectin ligands on leukocytes may be important to selectin recognition and affinity. However, the data provided in the Examples herein demonstrates that the *in vivo* administration of an anti-adhesion enzyme according to the inventive method results in appreciable reduction in inflammatory responses associated with cell adhesion via receptor/selectin adhesion ligand interactions, particularly acute inflammatory responses.

Because of the prevelance of fucosylated and sialilated selectin adhesion ligands in in leukocyte/endothelial cell adhesion events resulting in inflammation, the invention will be principally described in the context of reducing inflammation through administration of the anti-adhesion enzymes of the invention. However, the invention will be understood to not be so limited; i.e., the inventive method for administration of anti-adhesion enzymes may be utilized to inhibit adhesion of any cells wherein the adhesion occurs via formation of a bond between a carbohydrate residue in a cell surface ligand on the cell and a corresponding receptor which contains selectin.

A therapeutically effective dosage of the anti-adhesion enzyme will be administered by intravenous, intramuscular or other parenteral routes. Enzymatic activity in the plasma is verified and monitored by monitoring means such as spectrophometry as well as changes in clinical signs and symptoms.

Because when administered to a human the enzyme will preferably be a recombinant one of human origin, no immune response should be induced by its administration. Further, because a single enzyme molecule can cleave many different adhesion ligand residues, the potency of the composition is expected to be relatively high, and required dosage relatively low, as compared to prior art proposals for methods of inhibiting cell adhesion by leukocytes.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the pH activity curve of human α-L-fucosidase.

FIGURE 2 depicts the *in vitro* effect of substantially pure recombinant α-L-fucosidase in varying concentrations on adherence of neutrophils to human umbilical vein endothelial cells.

FIGURE 3 depicts the effect of sialidase on neutrophil content in lung tissue in rats injected with cobra venom factor (ARDS Model).

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

### A. Alternative Embodiments of Anti-adhesion Enzymes for Use in the Method of the Invention.

The anti-adhesion enzymes of the invention can be extracted from many mammalian tissues, including liver and placental tissues. For purposes of this application, however, the need for a consistent, high volume production source dictates that the recombinant form of the enzymes modified and produced as taught herein will be preferred.

#### 1. Production of recombinant and synthetic anti-adhesion enzymes.

(a) Human α-L-fucosidase.

The amino acid sequence shown in SEQ.ID.NO.1 is derived from sequence information obtained through isolation of several human α-L-fucosidase cDNA clones reported by Occhiodoro *et al.* at *Biochem. Biophys. Res. Comm.* 164:439-445 (1989), the disclosure of which is incorporated herein by this reference. Potential glycosylation sites are at nucleotides 754-756, 805-807 and 1147-1149. The full-length nucleotide sequence for α-L-fucosidase is publicly available from the GENBANK molecular sequence database under Accession No. M28099.

As noted above, the fucosidase whose amino acid sequence is depicted in SEQ.ID.NO.1 is preferred for use in the method of the invention for therapy in humans because it is of human origin. It will be appreciated by those skilled in the art, however, that other fucosidases are known and could be of potential therapeutic value in the inventive method, particularly for use in short-term treatment of acute inflammatory conditions where the immunogenicity of the enzyme may not be of as great concern as it would be if used in a treatment regime of longer duration.

Fucosidase activity in an enzyme to be used in the method of the invention may be determined according to conventional means for determining enzyme activity which are well-known in the art; for example, see Methods of Enzymology (New York Acad. Press, 1981). However, a particularly preferred and well-known method for determining fucosidase activity in an enzyme is disclosed in Zielke, *et al.*, *J. Lab. Clin. Med.* 79:164-169 (1972) and in Aldaheff, *et al.*, *J. Biol. Chem.* 250:7106-7113 (1975). These references are incorporated herein for the purpose of illustrating knowledge in the art regarding determination of fucosidase activity in a sample.

(b) Sialidases.

Sialidases have isolated from several vertebrate and microbial sources and cloned and the amino acid/nucleotide sequences reported in publicly accessible molecular sequence databanks such as GENBANK. For example, sialidases cloned from microbial sources include the gene from *Actinomyces viscosus* (GENBANK Accession Nos. S73643, S854402, S69682, X64360, S55807, S55811, S55812, S55814 and S55817); *Salmonella typhimurim LT2* (GENBANK Accession No.M55342); *Clostridium septicum* (expressed in *E.coli*; GENBANK Accession Nos. X63266, X54369, X54370, X54371, X54372, X54373, X54374, S79270, S79277, M64566); *Clostridium sordellii G12* (sequence not in public database; reported in Rothe, *et al.*, *J.Gen.Microbiol.* 135:3087-3096 (1989)), and *Clostridium perfringens* (GENBANK Accession No. Y00963; also reported in Roggentin, *et al.*, *FEBS Letters* 238:31-34 (1988)). Sialidase exists in humans but has not previously been cloned; however, there is substantial sequence homology among sialidases which should enable the production of probes for use in cloning human sialidase without undue experimentation (see, e.g., Rothe, *et al.*, *Mol.Gen.Genet.* 226:190-197 (1991) [identification of conserved sequences]; see also, Warner, *et al.*, *Carbohydr.Res.* 215:315-321 (1991) [method for distinguishing mammalian sialidases using inhibition kinetics studies and an unsaturated derivative of N-acetylneuraminic acid]).

With the available information regarding the amino acid and nucleotide sequences of various sialidases, sialidases for use in the inventive method can be readily synthesized or cloned by one of ordinary skill in the art through use of the methods described below without undue experimentation. To that end, each of the above-referenced references, including the related sialidase sequence data from GENBANK and equivalent sequence databases, are incorporated herein as examples of sialidases useful in this invention.

Purified non-human sialidases are also commercially available (from, for example, Sigma Chemical). However, as noted above with respect to the use of fucosidase, use of the human enzyme will be preferred, most preferably in recombinant form.

Sialidase activity in an enzyme to be used in the method of the invention may be determined according to conventional means for determining enzyme activity which are well-known in the art; for example, see Methods of Enzymology (New York Acad. Press, 1981).

(c) Methods for producing the recombinant and synthetic anti-adhesion enzymes of the invention.

Methods for producing the anti-adhesion enzymes of the invention are conventional cloning techniques which are well-known in the art. For example, the method of the invention encompasses the use of any catalytically active anti-adhesion enzyme which will cleave bonds between selectins and carbohydrate residues in selectin adhesion ligands, in particular sialidases and fucosidases. It is not intended, therefore, that the invention be considered as limited to the use of the specific enzymes whose amino acid sequences are referred to herein. Rather, functional derivatives of these enzymes and enzymes which are substantially similar in function are encompassed herein. Modifications of these enzymes to form chemical derivatives with additional characteristics, such as extended half-life, are also encompassed by this invention.

By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the anti-adhesion enzyme encoding DNA sequences of the present invention, includes any nucleotide subset of the molecule. A "variant" of such molecule refers to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule refers to a non-natural molecule which is substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is also said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Minor modifications of the primary amino acid sequences for the anti-adhesion enzymes of the invention may result in proteins which have substantially equivalent activity as compared to the specific anti-adhesion enzymes described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of an anti-adhesion enzyme (i.e., the ability to specifically cleave bonds between carbohydrate residues and selectins) still exists. Further, deletion of one or more amino acids can also result in a modification of the

structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which may not be required for the enzyme to exert the desired catalytic activity.

Similarly, a "functional derivative" of a polynucleotide sequence which encodes an anti-adhesion enzyme of the present invention includes "fragments", "variants", or "analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity. Thus, as used herein, "anti-adhesion enzyme" includes any functional derivative, fragments, variants, analogues, chemical derivatives which may be substantially similar to the anti-adhesion enzymes described herein and which possess similar activity.

DNA sequences for use in producing anti-adhesion enzymes other than those identified herein can be obtained by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to: 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening,

hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA.

The development of specific DNA sequences encoding the anti-adhesion enzymes of the invention can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

DNA sequences encoding the anti-adhesion enzymes can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, in other words when the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the genes encoding the anti-adhesion enzymes of the invention may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the appropriate genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host.

Polynucleotide sequences encoding the anti-adhesion enzymes of the invention can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having

eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences encoding the anti-adhesion enzymes of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the anti-adhesion enzymes of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

The polynucleotides encoding the anti-adhesion enzymes of the invention will preferably contain additional restriction sites to facilitate cloning which are appropriate to the recombinant expression vector to be used, which is Not-I for the preferred vectors. Examples of suitable eukaryotic recombinant expression vectors in addition to those used by Occhiodoro *et al.* are selectable eukaryotic expression vectors available from InVitrogen of San Diego, California (1992 product catalog) such as

pRc/CMV, pc DNAI/Neo or pRc/RSV, all of which contain a Not-I cloning site. The oligonucleotide for the Not-I enzyme has the sequence 5'-GCGGCCGC-3' and 3'-CGCCGGCG-5'.

These latter recombinant expression vectors are preferred for two reasons. First, they contain a neomycin selection marker for stable expression in mammalian cells. Second, use of a eukaryotic vector is generally preferred over prokaryotic vectors because use of a eukaryotic vector will allow for glycosylation of the recombinant enzyme. A prokaryotic recombinant expression vector for transfection into bacteria (preferably *E. coli*) may, however, be used. To this end, suitable prokaryotic expression vectors are available from InVitrogen of San Diego, California and include its pSE280 and pSE380 vector products.

Anti-adhesion enzymes of the invention whose amino acid sequences are known can also be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, (1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

An anti-adhesion enzyme clone having a desirable mutation as described further below may be amplified using a conventional polymerase chain reaction (PCR) and a primer pair corresponding to the 3' and 5' regions of the cDNA. The preferred method of amplification is the overlap extension PCR technique described by Ho, *et al., Gene* 77:51-59 (1989), the disclosure of which is incorporated herein by this reference. Generally, this technique accomplishes site-specific mutagenesis of the clone by utilizing a 3' primer to add the mismatched mutating bases (primer B in the Ho article, which is used with the 5' primer A in the first PCR cycle described). Amplification using the A and B primers yields an AB fragment. A second PCR cycle uses a primer (D) from the 3' end of the gene and a 5' mutated primer (C) complementary to primer B. The resulting amplification product (fragment CD) will overlap the AB fragment. When the AB and CD fragments are denatured, reannealed and amplified using the A and D primers, the resulting fusion product (AD) will contain the full-length cDNA sequence and the desired mutation.

It should be noted that it may be necessary to mutate any site of N-linked glycosylation in the anti-adhesion enzyme polypeptides of the invention to prevent mannosylation of the protein. This will prevent uptake of the recombinant enzyme by monocytes and macrophages that have receptors for mannose-phosphate, thus extending the half-life of the enzyme in a patient's circulation.

The overlap extension PCR technique described above may be used to eliminate the sites for N-linked glycosylation in the anti-adhesion enzyme produced according to the method described herein. For purposes of illustration, the polymerase chain reaction technique may be modified to change 1 to 3 of the asparagines in the sites of N-linked glycosylation to lysines or arginine (e.g., to change the AAT or AAC codons for asparagine to AGA or AGG, the codons for arginine).

Another suitable approach to modification of the lysines or arginines is described by Shaw in U.S. Patent No. 4,904,584 ("Site-Specific Homogenenous Modification of Polypeptides"), the disclosure of which is incorporated herein by this reference for purposes of illustration.

If, however, it is determined by assays described above that glycosylation of the enzyme enhances its desired functional activity, the interest in extending the half-life of the enzyme may be outweighed. In that instance, therefore, the sites for N-linked glycosylation will not be modified and a eukaryotic vector will be used to express the anti-adhesion enzyme.

Another useful mutation of the recombinant enzyme is one which will raise the pH optimum for the enzyme's activity from pH 5-5.5 to pH 7-7.4, the normal pH for human plasma (see, e.g., FIGURE 4 for the normal pH activity curve for $\alpha$-L-fucosidase in human serum [as determined at varying pH values in citrate-phosphate buffers for 30 minutes at 37°C]). This change to enhance the enzyme's activity in more alkaline environments is achieved by separately mutating single charged amino acids selected from the group consisting of lysine, arginine, histidine, glutamic and aspartic acid. Presently, the identity of the amino acid whose mutation will best enhance the activity of each of the anti-adhesion enzymes of the invention at pH 7-7.4 is not known, but can be systematically determined without undue experimentation by site-specific mutagenesis wherein a single charged amino acid will be substituted for with a different amino acid the product expressed as described above. An assay for enzyme activity at the desired pH will be performed according to the method disclosed in Zielke, *et al., J. Lab. Clin. Med.* 79:164-169 (1972), and the process repeated performing different non-cumulative mutations until the optimum pH for the desired enzyme activity has been raised to pH 7-7.4.

Once produced, the anti-adhesion enzyme will be purified by means well-known in the art to a preferred specific activity. For example, with respect to fucosidase, purification is best achieved by affinity chromatography using agarose to which aminocaproyl fucosamine has been coupled (suitable columns for use in this procedure are available from Sigma Chemical of St. Louis, Missouri or from Miles Laboratory of Naperville, Illinois).

More specifically, the tissue extract, transformed cell line supernatants, transformed bacterial lysates, or other source of the synthetic or recombinant fucosidase may be applied to an affinity column and washed with 10 mM phosphate buffer (pH 5.5) until all protein has been eluted. The column is then washed again with the phosphate buffer supplemented with 50 mM fucose available from Sigma) to elute the native or recombinant fucosidase, which is then concentrated using an ultrafiltration device (such as the ultrafiltration apparatus and microconcentrators available from Amicon of Danvers, Massachusetts) to a preferred concentration of 1.5 to 2.0 mg of enzyme per milliliter of buffer. Purity is confirmed according to means well known in the art for performance of SDS-polyacrylamide gel electrophoresis. All of the procedures described are performed at 2°-4°C and may be adapted for the purification of sialidases by one of ordinary skill in the art without undue experimentation.

The purified anti-adhesion enzyme will preferably have a specific activity of 25,000 - 30,000 units/mg of protein, wherein one unit (U) of enzyme activity is equivalent to the amount of enzyme which will hydrolyze 1 nmole of P-nitrophenyl-α-L-fucopyranoside or sialic acid substrate (available from Sigma) per minute at 37°C.

B. Preparation of Pharmaceutically Effective Compositions of Anti-adhesion Enzymes

Once the desired anti-adhesion enzyme has been produced and purified as described above, the enzyme is suspended in a buffer suitable for administration to humans, preferably isotonic phosphate buffered saline (pH 7.4) at a concentration of 1 to 10 mg enzyme/ml buffer, not to exceed 100 ml of buffer. The buffer may also be supplemented with stabilizers and/or anti-bacterial agents well known to those skilled in the art to maintain purity and extend shelf life of the anti-adhesion enzyme suspension.

In a preferred embodiment of the anti-adhesion enzyme composition, the enzyme will be modified to delay its clearance from the patient's bloodstream via the kidneys, thus extending the patient's exposure to the enzyme and reducing the number of times that the composition will have to be administered for effective treatment. To this end, the enzyme may be covalently attached to activated polyethylene glycol (PEG), which couples to the lysines and arginines in the protein.

Activated PEG is available from, for example, Sigma Chemical, in five different forms; an exemplary form for use in this embodiment is methoxypolyethylene glycol-succimidyl succinate. Whichever form of PEG is chosen, its selection should be made with a view toward chemically coupling at least 50% of the lysines and arginines in the protein.

The actual percentage of these amino acids which will be coupled in the final enzyme product will be that percentage which will help to maintain availability of the enzyme to the patient without destroying the catalytic activity of the enzyme. Generally, it can be expected that at least 50% of these amino acids will be coupled with PEG to avoid clearance of the enzyme by the patient's kidneys; the degree to which the useful life of the enzyme can be extended by coupling additional lysines and arginines to PEG

will depend on whether additional coupling significantly reduces enzyme activity. The point at which enzyme activity is significantly reduced can be determined by the assay procedures for enzyme activity described above (see, e.g., Aldaheff, *et al., J. Biol. Chem.* 250:7106-7113 (1975)).

Coupling of the lysines and arginines in the enzyme product to PEG may be achieved by use of several methods known to those skilled in the art (see, e.g., Savoca, *et al., Biochem. Biophys. Acta.* 578:47-53 (1979) and methods used in U.S. Patent 4,766,106). A suitable adaptation of these methods to conjugation of the anti-adhesion enzymes of the invention would be performed as follows. Approximately 25 mg of purified recombinant enzyme is dissolved in 5 ml of 50 mM phosphate buffered saline (pH 7.2). The dissolved enzyme is then incubated with approximately 500 mg of activated polyethyleneglycol (such as methoxypolyethylene glycol-succinimidye succinate, available from Sigma), then isolated by means known in the art (see, e.g., Abuchowski *et al.*, "Enzymes as Drugs", John Wiley and Sons (1981)).

C. **Clinical Administration of the Anti-adhesion Enzyme Composition.**

The anti-adhesion enzyme composition will be administered to the mammal in a therapeutically effective dosages, preferably by intra-arterial infusion. In this context, a "therapeutically effective dosage" refers to that amount of anti-adhesion enzyme which will produce a detectable reduction in inflammation without substantial toxicity to the patient. Generally, this dosage will comprise about 0.6 to 234 U/kg of the patient's body weight.

Typically, the infusion will be performed for an extended period of up to about 4 hours, although those of ordinary skill in the clinical art will know or be able to readily determine how to adjust the course of treatment to suit the therapy needs of particular patients. Administration of this dosage will preferably be repeated at intervals of two times per week to once a month, depending on the patient's

presenting condition and responsiveness to the therapy. Enzymatic activity in the plasma is verified and monitored by spectrophotometry according to means well-known in the art; changes in clinical signs and symptoms will provide a further basis for assessment of functional enzyme activity.

Based on the role of leukocytes in endothelial cell activation, it is expected that the anti-adhesion enzyme suspension will be effective in therapy for acute inflammatory conditions associated with septic and endotoxin shock, post-pump syndrome following cardiopulmonary bypass, the acute rejection of transplanted organs, reprofusion injury following myocardial infarction or stroke and peripheral vascular injuries. It is also expected to be of therapeutic value in treatment of chronic inflammation associated with rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis and inflammatory iritis.

Based on *in vitro* exposure of tumor cells having metastasic capability (rat mammary adenocarcinoma cell line R13762) to bovine fucosidase (see data reported in Wright, *et al. J. Cell. Biochem.* 37:49-59, (1988)) it is also expected that the human fucosidase composition and, possibly, the sialidase composition disclosed herein will be of value in limiting metastasis of tumor cells including, but not limited to, those associated with cancers of the breast, colon, prostate, lung, stomach and pancreas.

It will be appreciated by those skilled in the art that there may be various ways to practice this invention. These examples should, therefore, be regarded as illustrative rather than exhaustive of those possibilities.

## EXAMPLE I

## PROTOCOL FOR MEASUREMENT OF *IN VIVO* Anti-adhesion RESPONSE TO FUCOSIDASE COMPOSITION IN RABBIT MODEL

A substantially pure α-L-fucosidase composition was prepared as discussed above without coupling to PEG or mutation to enhance optimal pH activity or to prevent mannosylation. In this context, "substantially pure" refers to a fucosidase composition which consists essentially of catalytically active fucosidase, but may also contain catalytically active sialidase. The pRc/CMV or pcDNAI/Neo eukaryotic expression vectors available from InVitrogen of San Diego, California were used to express the enzyme.

The fucosidase composition was injected in a dosage of 0.01-0.1 mg/kg of body weight into New Zealand white rabbits of 2-3 kg body weight. One hour later, carrageenan (available from Sigma in a preparation of 2.5 mg carrageenan/0.25 ml water) was injected into the pleural cavity of each rabbit. Four hours following the carrageenan injection, the rabbits were sacrificed and the plural cavity opened. The plural exudate was carefully removed by pipetting and assayed for total volume of exudate (in milliliters) and for total cellular influx.

The effectiveness of the suspension is demonstrated by a ≥ 25% reduction in total exudate volume in the pleural cavity and in cellular influx, as compared to controls injected only with carrageenan. This assay can be performed at with any site of extravascular inflammation, such as in the skin of the ear. It should be noted, however, that use of rats in lieu of rabbits or other mammalian models is not recommended because fucose apparently does not play a significant role in rat inflammation responses.

## EXAMPLE II

## *IN VITRO* INHIBITION OF NEUTROPHIL ADHERENCE TO HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVEC) available from Clonetics of San Diego, California were propagated in microtiter plates in EGMUV medium (also available from Clonetics) which were supplemented with 2% fetal bovine serum and used during the first three passages. To increase their adhesiveness, the HUVEC cells were treated with recombinant interleukin-1 (IL-1) (R&D Systems, Minneapolis, MN) at a concentration of 5 ng/ml for four hours prior to analysis.

Neutrophils were isolated from human peripheral blood by Ficoll-Hypaque sedimentation according to means well known in the art. The cell pellet was treated for 10 seconds with water to lyse erythrocytes, and then was washed two times in Hank's balanced salt solution (HBSS) supplemented with 1% human plasma. The isolated neutrophils were incubated with bovine kidney fucosidase at the concentrations indicated in FIGURE 2 for one hour in HBSS with 1% autologous plasma. The cells were then washed and then added at a density of 200,000 cells/ml to the HUVEC monolayers. After 45 minutes at 37°C, unadhered neutrophils were removed gently with a pipette followed by rinsing with warm HBSS containing 1% plasma. Then 0.1% Triton X-100 is added to the cultures to lyse the neutrophils.

After an overnight incubation, released neutrophil myeloperoxidase was measured spectrophotometrically by the oxidation of diaminobenzidine with $H_2O_2$ in the presence of the enzyme, using a commercial assay (the kit for which is available from Sigma, St. Louis). Since myeloperoxidase is present in neutrophils and not endothelial cells, the total enzyme level is directly proportional to the number of adherent neutrophils in each culture. As indicated in FIGURE 2, pretreatment of the neutrophils with the fucosidase composition blocked their ability to adhere to HUVEC.

## EXAMPLE III

## PREVENTION OF NEUTROPHIL ENTRY INTO LUNG TISSUE IN AN ADULT RESPIRATORY DISTRESS SYNDROME MODEL

A sialidase composition of microbial origin (*Clostridium*) was prepared as described elsewhere above. To create a model for the acute inflammatory condition known as adult respiratory distress syndrome (ARDS), "COBRA VENOM FACTOR" (CVF), a tradenamed product of Calbiochem of San Diego, was administered to rats as follows. In this model of ARDS, CVF activates the complement system. The resulting formation of the complement factor C5A induces neutrophil activation. The activated neutrophils accumulate in the lung vasculature, causing tissue damage.

CVF was diluted in phosphate buffered saline (PBS) to 32 $\mu$/ml and dialyzed overnight at 4°C in PBS. The final concentration of the CVF was 22.8 $\mu$/ml. The sialidase was resuspended in PBS to a volume of 50 $\mu$/ml and injected into the rat's tails in volume of 200 $\mu$l of PBS. Control rats received injections of 0.5 ml PBS.

After 80 minutes, secondary injections were made of either PBS (control rats) or 11.4 units of CVF. Thirty minutes later, rats were anaesthetized with an intraperitoneal injection of ketamine hydrochloride. Rat lung vasculature was then perfused with 10 ml PBS injected in the right ventricle with the inferior vena cava and superior vena cava clamped.

Lung tissue was dissected out with a specimen fixed in formalin for histological study and a second specimen place in PBS for myeloperoxidase activity assay. Rat lungs in PBS were frozen at -20°C for 2 days. After 2 days, rat lung specimens were thawed in a 37 °C circulating water bath. Approximately 500 mg of lung tissue was minced to fine pieces approximately 1 $mm^2$ using sterile scalpel and forceps. Lung pieces were then sonicated at a setting of 4.5 on a microtip sonicator for 45, 30 and 10 second intervals. After sonication, 1.5 ml aliquots were transferred to microcentrifuge tubes and centrifuged at 4°C for 3 minutes at 16,000 RCF.

Supernatants were transferred to new microcentrifuge tubes and assayed for myeloperoxidase activities as described elsewhere above.

The result of this assay demonstrates that the rats which received the sialidase injections were able to fend off the neutrophil entry into lung tissue which characterizes the inflammatory response in ARDS. The data from the sialidase injected mice and the control mice are shown in FIGURE 3.

It will be appreciated that modifications to this invention, which is defined by the appended claims, may be made without departing from its spirit or scope.

## SUMMARY OF SEQUENCES

SEQUENCE ID NO 1 is the polynucleotide sequence which will encode human α-L-fucosidase, including the signal peptide (bases 19 to 84) and the mature protein (bases 85 to 1401).

SEQUENCE ID NO 2 is the deduced amino acid sequence for human α-L-fucosidase, including the signal peptide and mature protein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Regents of the University of California, *et al.*

(ii) TITLE OF INVENTION: METHOD FOR INHIBITION OF CELL ADHESION TO RECEPTORS CONTAINING SELECTINS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SPENSLEY HORN JUBAS & LUBITZ
(B) STREET: 1880 CENTURY PARK EAST, FIFTH FLOOR
(C) CITY: LOS ANGELES
(D) STATE: CALIFORNIA
(E) COUNTRY: US
(F) ZIP: 90067

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE: 21-DEC-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HOWELLS, STACY L.
(B) REGISTRATION NUMBER: 34,842
(C) REFERENCE/DOCKET NUMBER: FD-2929

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 455-5100
(B) TELEFAX: (619) 455-5110

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2035 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: ALPHA-L-FUCOSIDASE

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 19..1401

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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       125                 130                 135

10 TGG CCG AGT CCT GTG TCT TGG AAC TGG AAC TCC AAA GAC GTG GGG CCT      483
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   140                 145                 150                 155

   CAT CGG GAT TTG GTT GGT GAA TTG GGA ACA GCT CTC CGG AAG AGG AAC      531
   His Arg Asp Leu Val Gly Glu Leu Gly Thr Ala Leu Arg Lys Arg Asn
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   ATC CGC TAT GGA CTA TAC CAC TCA CTC TTA GAG TGG TTC CAT CCA CTC      579
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   His Trp Pro Glu Asn Gly Val Leu Asn Leu Glu Ser Pro Ile Thr Thr
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Val Lys

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CAGTTGTCAC CAACAGTCTT TCTGCAAAGG GCAGGAGAGC TTTGGGGGAA AGGAAAAGGC    1871

TTACCAGGCT GCTATGGTCA ACTCTTCAGA AATTTTCAGA GCAATCTAAA AGCGCCAAAA    1931

TTCGCTATGT TTACAGTGAT ACTATTAAGA AAATGAATGT GATTCTGCTC TGTCTTTTTA    1991

AGTATGATCA AATAAAAAAT TTGTACATCA CAATCATTTC TACC                     2035
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 461 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Leu Ser Trp Leu Tyr Asn Asp Ser Pro Val Lys Asp Glu Val Val Val
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Lys Gly Leu Phe Ile Ser Leu Pro Gln Leu Pro Pro Ser Ala Val Pro
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Ala Glu Phe Ala Trp Thr Ile Lys Leu Thr Gly Val Lys
450 455 460

## CLAIMS

1. A method for inhibition of adhesion of cells to receptors containing selectins in a mammal comprising:

administration to the mammal of a pharmaceutically acceptable composition containing a therapeutically effective dosage of at least one anti-adhesion enzyme, which enzyme will specifically cleave a carbohydrate residue in a selectin adhesion ligand for selectin-containing receptors on endothelial cells.

2. The method according to Claim 1 wherein the residue is sialilated.

3. The method according to Claim 2 wherein the anti-adhesion enzyme comprises a sialidase.

4. The method according to Claim 1 wherein the residue is fucosylated.

5. The method according to Claim 1, wherein the anti-adhesion enzyme comprises a fucosidase.

6. The method according to Claim 1, wherein the composition contains sialidase and fucosidase.

7. The method according to Claim 3, wherein the sialidase is of human origin.

8. The method according to Claim 5, wherein the fucosidase is human $\alpha$-L-fucosidase.

9. The method according to Claim 6, wherein the fucosidase and sialidase are of human origin.

10. The method according to Claim 3, wherein the sialidase is a recombinant enzyme.

11. The method according to Claim 5, wherein the fucosidase is a recombinant enzyme.

12. The method according to Claim 1, wherein the anti-adhesion enzyme is modified to bring the pH requirement for the optimal activity of the enzyme to within the range of pH 7-7.4.

13. The method according to Claim 1, wherein the anti-adhesion enzyme is modified to eliminate the production thereby of sites for N-linked glycosylation.

14. The method according to Claim 1, wherein the anti-adhesion enzyme is chemically conjugated to polyethylene glycol.

15. The method according to Claim 1, wherein the therapeutic efficacy of the anti-adhesion enzyme is monitored by assay of the patient's plasma by means for detecting the extent of any inflammatory response.

16. The method according to Claim 1, wherein the mammal is a human.

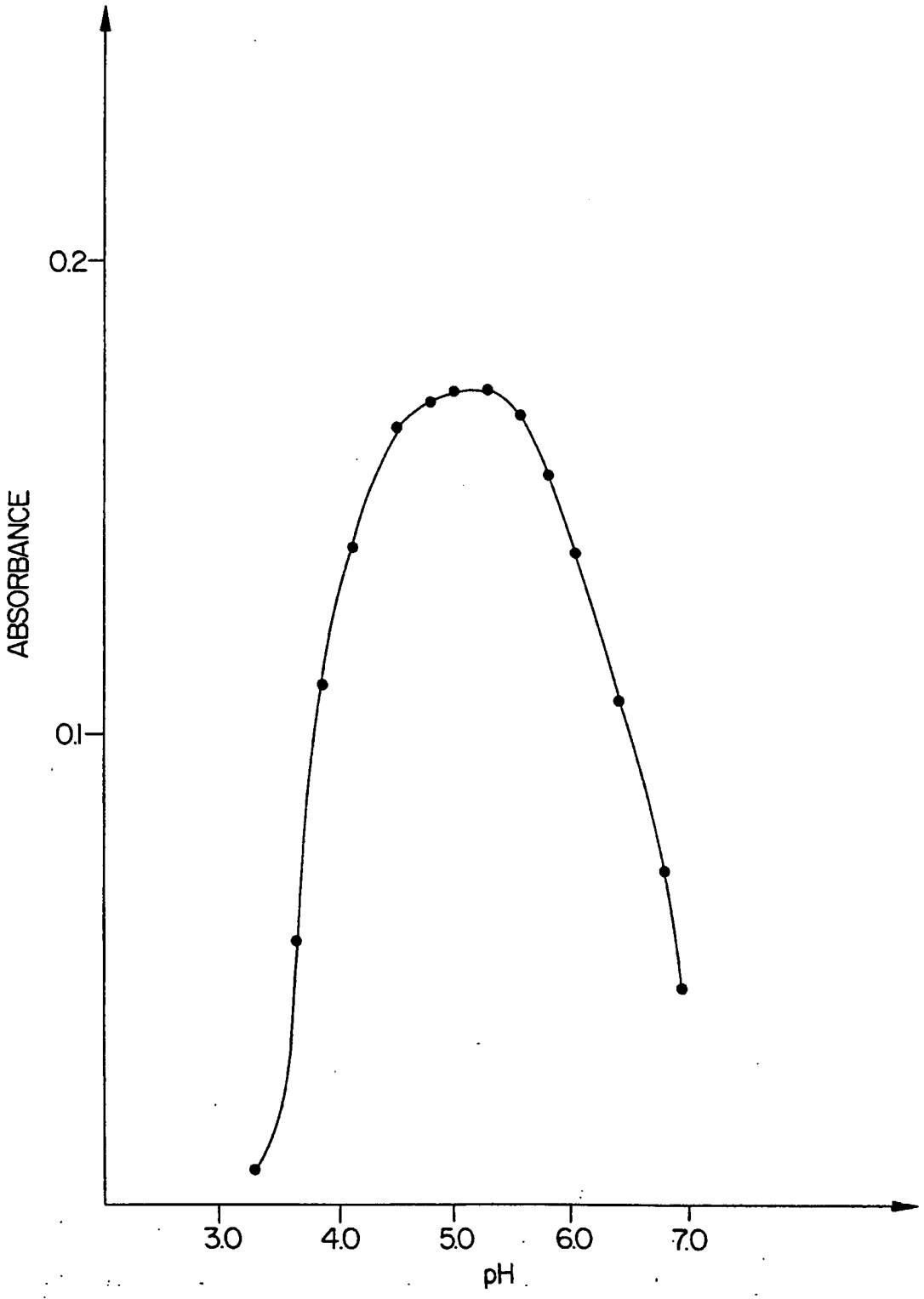


FIG. 1

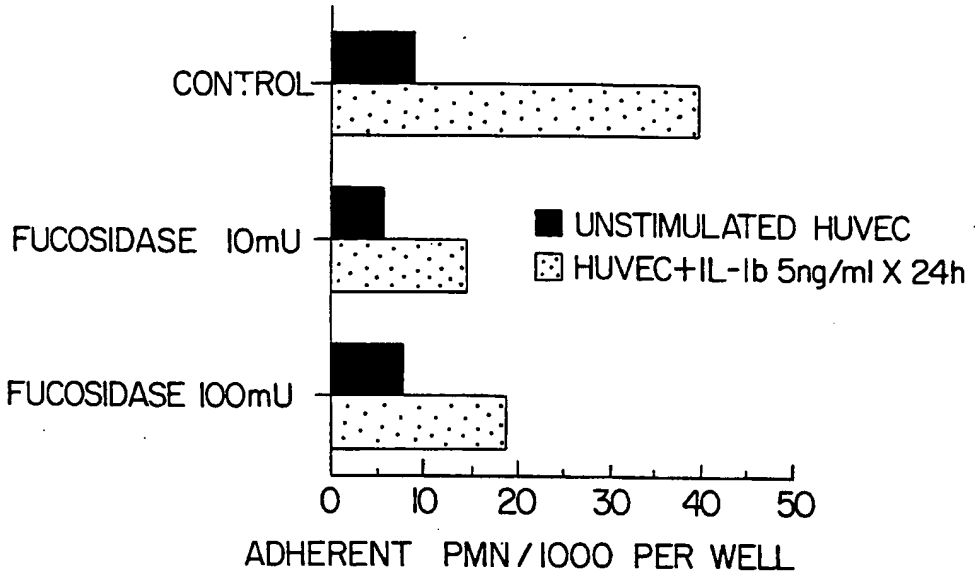
CONTROL
FUCOSIDASE 10mU
FUCOSIDASE 100mU
UNSTIMULATED HUVEC
HUVEC+IL-1b 5ng/ml X 24h
0
10
20
30
40
50
ADHERENT PMN / 1000 PER WELL

FIG. 2

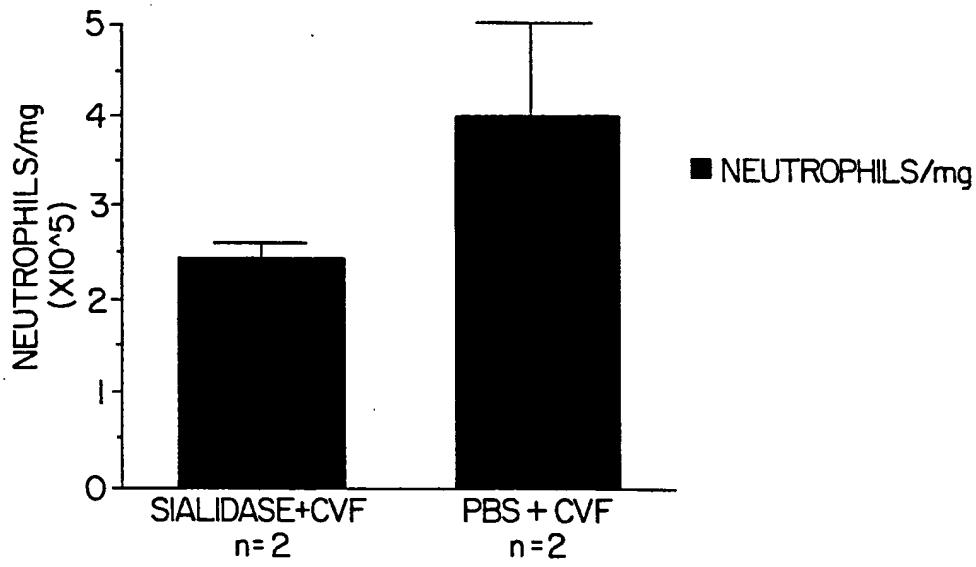
NEUTROPHILS/mg (X10^5)
0
1
2
3
4
5
SIALIDASE+CVF n=2
PBS + CVF n=2
NEUTROPHILS/mg

FIG. 3

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12464

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 37/54; C12N 9/24
US CL :514/2, 12; 435/200

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12; 435/200

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | J. Cell Biol., Vol. 115, No. 2, issued October 1991, Zhou et al., "The Selectin GMP-140 Binds To Sialylated, Fucosylated Lactosaminoglycans On Both Myeloid And Nonmyeloid Cells", pages 557-564, see entire document. | 1-4<br>1-16 |
| Y | Proc. Natl. Acad. Sci. USA, Vol. 88, issued February 1991, Tiemeyer et al., "Carbohydrate Ligands For Endothelial-Leukocyte Adhesion Molecule 1", pages 1138-1142, see entire document. | 1-16 |
| Y | US, A, 4,904,584 (SHAW, G.) 27 February 1990, see entire document. | 1-16 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
08 MARCH 1994

Date of mailing of the international search report
MAR 21 1994

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12464

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Biochem. Biophys. Res. Commun., Vol. 164, No.1, issued 16 October 1989, Occhiodoro et al., "Human α-L-Fucosidase: Complete Coding Sequence From cDNA Clones", pages 439-445, see entire document. | 1-16 |
| Y | The Lancet, Vol. 2, issued 04 October 1986, Taylor et al., "Enzyme Replacement In Nervous Tissue After Allogenic Bone-Marrow Transplantation For Fucosidosis In Dogs", pages 772-774, see entire document. | 1-16 |
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS - USPAT, JPOABS
Dialog - files 5, 35, 73, 155, 203, 351, 357, and 144,
EMBL_NEW, GenBank 80, GenBank-NEW, N-GeneSeq 13, UEMBL 37_80, VectorBank 6.4

Search terms icam-1, elam-1, fucosidase?, fucosylate?, sialylate?, sialidase?, inhibit?, adhesion, fucosidosis, inflamm?